Effects of cholesterol feeding on synthesis and metabolism of cholesterol and bile acids in germfree rats

Bengt E. Gustafsson,[1] Bo Angelin, Kurt Einarsson, and Jan-Åke Gustafsson

Department of Germfree Research, Department of Medicine,[2] Serafimerlasarettet, and
Department of Chemistry,[3] Karolinska Institutet, Stockholm, Sweden

Abstract The aim of the present investigation was to study the influence of cholesterol feeding on cholesterol synthesis and fecal excretion of bile acids in germfree rats. Four germfree rats were fed a basal diet containing 0.004% cholesterol and four germfree rats received the same diet supplemented with 0.4% cholesterol for 2 weeks. Cholesterol synthesis was studied by assaying the HMG CoA reductase activity in the liver microsomal fraction. Cholesterol feeding decreased the HMG CoA reductase activity from 28.5 ± 6.6 (mean ± SEM) to 9.1 ± 0.7 pmol/mg protein per min. In another experiment four germfree rats received the basal diet and four germfree rats the cholesterol-enriched diet. After 6 weeks feces were collected in two 4-day pools for analysis of bile acids. The main fecal bile acids were cholic acid and β-muricholic acid (a metabolite of chenodeoxycholic acid), comprising more than 95% of total bile acids. Cholic acid was increased from 3.9 ± 0.2 to 9.9 ± 1.2 mg/kg body weight per day and β-muricholic acid from 6.6 ± 0.5 to 21.8 ± 3.1 mg/kg body weight per day. The percentage of cholic acid decreased from 37.1 ± 1.1 to $31.2 \pm 1.0\%$. In conclusion, germfree rats like conventional rats have the ability to compensate for an increased input of dietary cholesterol by inhibition of cholesterol synthesis and stimulation of bile acid synthesis. The synthesis of chenodeoxycholic acid (implied from the fecal excretion of β-muricholic acid) is stimulated to a greater extent than that of cholic acid.

Supplementary key words HMG CoA reductase · cholic acid · chenodeoxycholic acid · β-muricholic acid

The hepatic cholesterol concentration in germfree rats has been reported to be higher than in conventional rats (1, 2). This accumulation of cholesterol in germfree animals may be explained by a more efficient absorption from the intestine (3) and by a decreased fecal excretion of bile acids and neutral steroids (4, 5). The synthesis of cholesterol, on the other hand, is depressed in germfree rats compared with conventional rats (6). Addition of moderate amounts of cholesterol to the diet leads to higher levels of tissue cholesterol in germfree rats than in conventional rats (7). Thus, it would seem as if the intestinal microflora to some extent protects against increased tissue cholesterol levels in response to dietary cholesterol.

Feeding a high cholesterol diet to conventional rats increases the absorption of cholesterol (8, 9). In answer to this high cholesterol input, the hepatic cholesterol synthesis is inhibited and the catabolism of cholesterol to bile acids is increased (9–11). These mechanisms are not completely compensatory and a certain accumulation of cholesterol occurs in the liver (9, 12). The question may now be asked whether the higher hepatic concentration of cholesterol in livers of germfree rats than in livers of conventional rats may be explained by a deficient compensation in germfree rats to the augmented intestinal input of cholesterol. We have recently shown that the 7α-hydroxylase, which is a rate-determining enzyme in the formation of bile acids from cholesterol, is enhanced to about the same degree in germfree and in conventional rats during cholesterol feeding, indicating that germfree rats would have the ability to increase the synthesis of bile acids (2, 13). However, these in vitro experiments were not correlated with any simultaneous measurements of fecal bile acids.

To obtain further insight into the mechanisms behind the differences in hepatic cholesterol concentration between germfree and conventional rats, we have administered cholesterol to germfree rats

---

and determined the activity of HMG CoA reductase, which is a rate-determining enzyme in cholesterol synthesis. In addition, the effect of cholesterol feeding on the excretion of fecal bile acids in germfree rats was studied. No attempt was made to perform comparative studies on the effect of cholesterol feeding on the excretion of fecal bile acids in conventional rats. Such a comparison would be very complicated, because, in these animals, bile acids are metabolized to a spectrum of secondary bile acids and, possibly, also to nonsteroidal derivatives (14, 15) by the intestinal microorganisms. This makes quantification of total fecal bile acids complicated. In feces from germfree rats, on the other hand, cholic acid and β-muricholic acid comprise more than 95% of the total bile acids, which simplifies a quantitative analysis. For these reasons, cholesterol effects on fecal bile acids were studied only in germfree rats.

MATERIALS

[3-^{14}C]HMG CoA (sp act 20 μCi/mg) and DL[5-^{3}H (N)]mevalonic acid (dibenzylethylene-diamine salt, sp act 25 μCi/mg) were purchased from New England Nuclear Corp., Boston, MA. The radioactive HMG CoA was diluted with unlabeled material, obtained from P-L Biochemicals, Inc., Milwaukee, WI, to yield a sp act of 1.45 μCi/mg. Unlabeled DL-mevalonic acid lactone, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, MO.

METHODS

Animals and preparation of microsomes

Germfree male rats of the Sprague-Dawley strain weighing 200–300 g were used. They were reared according to the technique of Gustafsson (16, 17). They were fed a semisynthetic diet ad libitum with 10% (w/w) arachis oil as the source of fat and supplemented with 0.4% cholesterol (17). Control groups of germfree rats were given the same diet as the experimental groups but with the exclusion of additional cholesterol. This latter diet contained 0.004% cholesterol.

In one experiment, four germfree rats received the basal diet and four germfree rats the cholesterol-enriched diet for 14 days. They were killed by a blow on the neck immediately after they had been taken out of the germfree isolators at 9 AM. The livers were removed quickly and chilled on ice. Liver ho-

mogenates (10%, w/v) were prepared in a medium containing 0.3 M sucrose, 0.075 M nicotinamide, 0.002 M EDTA, and 0.02 M mercaptoethanol, using a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle. The homogenate was centrifuged at 20,000 g for 15 min. The microsomal fraction was obtained by centrifuging the 20,000 g supernatant fluid at 100,000 g for 70 min. The microsomes were washed with the homogenizing medium once and centrifuged again at 100,000 g for 30 min. They were then suspended in the homogenizing medium in a volume corresponding to that of the 20,000 g supernatant fluid. The protein concentration was determined by the method of Lowry et al. (18).

In another experiment four germfree rats received the basal diet and four germfree rats the cholesterol-enriched diet. After the rats were fed the experimental diets for 6 weeks, feces were collected in two 4-day pools and were stored at −20°C until analyzed. This long feeding time was chosen since germfree rats have a very long transit time of intestinal contents; consequently, following a change in diet, it will take several weeks until a new steady state in bile acid metabolism is reached and the quantity of the fecal bile acids excreted corresponds to the amount of newly synthesized bile acids (19).

Assay of HMG CoA reductase activity

The assay system used was similar to that described by Shefer et al. (20). The complete system contained in a volume of 0.85 ml: 0.2 ml of microsomal fraction; phosphate buffer, pH 7.2, 100 mM; NADP, 3 mM; glucose-6-phosphate, 10 mM; glucose-6-phosphate dehydrogenase, 5 enzyme units, [3-^{14}C]-HMG CoA, 0.2 mM. The incubation was carried out for 15 min at 37°C. It was stopped by the addition of 0.1 ml of 5 M HCl. Tritium-labeled mevalonic acid (0.01 μCi) and 3 mg of unlabeled mevalonic acid lactone were added as internal standards and the incubation was further shaken for 30 min at 37°C. After cooling to room temperature, 0.5 ml of absolute ethanol was added and the mixture was dried over anhydrous Na$_2$SO$_4$. The solution was extracted three times with 3 ml of ethyl ether. The ethyl ether fractions were combined, dried over anydrous Na$_2$SO$_4$ and evaporated. The residue of the extract was dissolved in acetone and subjected to TLC with benzene–acetone 1:1 (v/v) as solvent. The thin-layer plate was sprayed with a 0.2% (w/v) solution of 2,7-dichlorofluorescein in methanol and the band corresponding to mevalonic acid lactone was located under UV light. This band was scraped off into a counting vial. The radioactivity was determined in a Packard liquid scintillation spectrometer model

3003, using Instagel as a scintillator liquid. Corrections were made for quenching and for losses during extraction and TLC by the internal standard. The recovery of tritium-labeled internal standard was about 50%.

Analysis of fecal bile acids

Feces were disintegrated in water and refluxed in 70% (v/v) aqueous ethanol for 2 hr. After filtration, the residue was refluxed in chloroform–methanol 1:1 (v/v) for 2 hr. The extracts were combined and an aliquot (1/10 or 1/5) was further hydrolyzed with 1 M KOH in 50% aqueous ethanol for 12 hr at 110°C. The saponification mixture was extracted with hexane, which was discarded. The mixture was then acidified with 6 M HCl and extracted with ethyl acetate. The ethyl acetate was washed with water until neutral and the solvent was evaporated. [^{14}C]Cholic acid was used as an internal standard in the extraction procedure for the correction for losses. The recovery amounted to about 85%.

The residue of the ethyl acetate extract was methylated with diazomethane, silylated, and analyzed by GLC using 1% HiEff 8 BP as the stationary phase.

RESULTS

HMG CoA reductase activity

The amount of formed mevalonic acid lactone was expressed as pmol/mg protein per min. In the germfree rats fed the basal diet the reductase activity averaged 28.5 ± 6.6 (mean ± SEM) pmol/mg protein per min (**Table 1**). In the cholesterol-fed rats the reductase activity was depressed to 9.8 ± 0.7 pmol/mg protein per min ($P < 0.05$).

Fecal bile acids

The two dominating bile acids in feces from germfree rats were cholic acid and β-muricholic acid. These two bile acids comprised more than 95% of total fecal bile acids in both dietary groups of rats. Small amounts of chenodeoxycholic acid and α-muricholic acid were identified but they were not included in the calculations of total bile acids. **Table 2** summarizes the results when germfree rats were given dietary cholesterol. Cholic acid increased 2.5 times from 3.9 ± 0.2 mg to 9.9 ± 1.2 mg. β-Muricholic acid similarly increased threefold from 6.6 ± 0.5 mg to 21.8 ± 3.1 mg. The sum of cholic acid and β-muricholic acid increased threefold. The percentage of cholic acid decreased significantly ($P < 0.005$) from 37.1 ± 1.1% to 31.2 ± 1.0%.

TABLE 1. The influence of cholesterol feeding on the activity of HMG CoA reductase in liver microsomes from germfree rats

	Mevalonic Acid Lactone Formed	
Rat	Control Rats	Cholesterol-Fed Rats
	pmol/mg protein per min [a]	
1	46.8	9.4
2	18.7	9.2
3	29.2	10.8
4	19.3	6.9
Means ± SEM	28.5 ± 6.6	9.1 ± 0.7
Significance[b] of differences	$P < 0.05$	

[a] Values are the means of two determinations in each rat.
[b] Student's *t* test.

DISCUSSION

The response to dietary cholesterol is different in various species. Feeding a high cholesterol-containing diet to man causes an increased absorption, a depressed endogenous synthesis of cholesterol, and an enhanced fecal excretion of neutral steroids (21, 22). Feeding cholesterol to the rat also increases the absorption of cholesterol and inhibits cholesterol synthesis in the liver, whereas the fecal excretion of neutral steroids is only slightly increased (8–10). The rat makes up for a high input of cholesterol mainly by an enhanced breakdown of cholesterol to bile acids (9, 11). The present results show that germfree rats, in conformity with conventional rats, compensate an increased intake of cholesterol by inhibition of hepatic cholesterol synthesis and increased excretion of fecal bile acids.

By far the dominating fecal bile acids in germfree rats are cholic acid and β-muricholic acid (4, 23). The major bile acids formed in the liver are cholic acid and chenodeoxycholic acid (for a review, see ref. 24). The first step in the conversion of cholesterol into bile acids is 7α-hydroxylation, which is rate determining. 5-Cholestene-3β,7α-diol is further converted to 7α-hydroxy-4-cholesten-3-one which is a key intermediate in bile acid biosynthesis. In cholic acid formation 7α-hydroxy-4-cholesten-3-one is 12α-hydroxylated to yield 7α,12α-dihydroxy-4-cholesten-3-one. In chenodeoxycholic acid synthesis 7α-hydroxy-4-cholesten-3-one is either directly 26-hydroxylated or further reduced to 5β-cholestane-3α,7α-diol which is 26-hydroxylated. Chenodeoxycholic acid is 6β-hydroxylated to α-muricholic acid which is converted to β-muricholic acid.

In a previous study we investigated the influence of cholesterol feeding to germfree rats on some hydroxy-

TABLE 2. The influence of cholesterol feeding on the fecal excretion of bile acids in germfree rats[a]

Rats	Cholic Acid	β-Muricholic Acid	Total Bile Acids	% Cholic Acid of Total Bile Acids
	mg/kg body weight/day			
Control rats[b]	3.9 ± 0.2	6.6 ± 0.5	10.5 ± 0.6	37.1 ± 1.1
Cholesterol-[b] fed rats	9.9 ± 1.2	21.8 ± 3.1	31.7 ± 4.3	31.2 ± 1.0
Significance of differences[c]	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.005$

[a] Values are the means ± SEM of eight fecal pools collected from four rats.
[b] The weight given was the same in the control and cholesterol-fed groups.
[c] Student's t test.

lases involved in the formation and metabolism of bile acids (2). The 7α-hydroxylation of cholesterol was stimulated 2.5 times, the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one was inhibited about 30%, and the 6β-hydroxylation of lithocholic acid was stimulated almost twofold. The 6β-hydroxylase involved in this reaction has the same properties as that active on chenodeoxycholic acid. In the present study we found that cholesterol-fed rats had a three-fold greater excretion of fecal bile acids compared to those fed a regular diet. Furthermore, the excretion of β-muricholic acid was increased more than that of cholic acid. These results fit very well with the above mentioned in vitro data, i.e., cholesterol feeding stimulates 7α-hydroxylation of cholesterol and 6β-hydroxylation of chenodeoxycholic acid, whereas 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one is inhibited.

Previous investigations have given evidence for a certain compartmentation of cholesterol in rat liver (25–27). Cholic acid seems to be formed from newly synthesized cholesterol to a greater extent than chenodeoxycholic acid. Our results are in agreement with such an assumption. The synthesis of chenodeoxycholic acid was stimulated more than that of cholic acid when the input of exogenous cholesterol to the liver was increased and the endogenous formation of cholesterol was depressed.

In conclusion, the present investigation has shown that germfree rats have compensatory mechanisms that are activated when the animals are given increased amounts of cholesterol, i.e., a reduced HMG CoA reductase activity in vitro and an increased formation of bile acids in vivo. Previous reports have shown that germfree rats accumulate more tissue cholesterol than conventional rats (1, 2, 7). Whether this difference between conventional and germfree rats depends on quantitatively more sufficient compensatory mechanisms in conventional rats or may be ascribed to a more efficient absorption of

cholesterol from the intestine in germfree rats (3) cannot be decided from the present investigation.▮▮

REFERENCES

1. Kellogg, T. F., and B. S. Wostmann. 1968. Lipid metabolism. *In* The Germfree Animal in Research. M. E. Coates, editor. Academic Press, New York. 181–196.
2. Gustafsson, B. E., K. Einarsson, and J-Å. Gustafsson. 1975. Influence of cholesterol feeding on liver microsomal metabolism of steroids and bile acids in conventional and germ-free rats. *J. Biol. Chem.* **250:** 8496–8502.
3. Wostmann, B. S. 1973. Intestinal bile acids and cholesterol absorption in the germfree rat. *J. Nutr.* **103:** 982–990.
4. Kellogg, T. F., and B. S. Wostmann. 1969. Fecal neutral steroids and bile acids from germfree rats. *J. Lipid Res.* **10:** 495–503.
5. Kellogg, T. F. 1974. Steroid balance and tissue cholesterol accumulation in germfree and conventional rats fed diets containing saturated and polyunsaturated fats. *J. Lipid Res.* **15:** 574–579.
6. Einarsson, K., J-Å. Gustafsson, and B. E. Gustafsson. 1977. Hepatic 3-hydroxy-3-methylglutaryl CoA reductase activity in germfree rats. *Proc. Soc. Exp. Biol. Med.* **154:** 319–321.
7. Kellogg, T. F., and B. S. Wostmann. 1969. The response of germfree rats to dietery cholesterol. *Adv. Exp. Med. Biol.* **3:** 293–295.
8. Borgström, B. 1968. Quantitative aspects of the intestinal absorption and metabolism of cholesterol and β-sitosterol in the rat. *J. Lipid Res.* **9:** 473–481.
9. Raicht, R. F., B. I. Cohen, S. Shefer, and E. H. Mosbach. 1975. Sterol balance studies in the rat. Effects of dietary cholesterol and β-sitosterol on sterol balance and rate-limiting enzymes of sterol metabolism. *Biochim. Biophys. Acta.* **388:** 374–384.
10. Weis, H. J., and J. M. Dietschy. 1969. Failure of bile

acids to control hepatic cholesterogenesis: evidence for endogenous cholesterol feedback. *J. Clin. Invest.* **48:** 2398–2408.

11. Wilson, J. D. 1964. The quantification of cholesterol excretion and degradation in the isotopic steady state in the rat: the influence of dietary cholesterol. *J. Lipid Res.* **5:** 409–417.

12. Beher, W. T., G. D. Baker, and D. G. Penney. 1963. A comparative study of the effects of bile acids and cholesterol on cholesterol metabolism in the mouse, rat, hamster and guinea pig. *J. Nutr.* **79:** 523–530.

13. Mosbach, E. H., and G. Salen. 1974. Bile acid biosynthesis. Pathways and regulation. *Amer. J. Dig. Dis.* **19:** 920–929.

14. Ahrens, E. H. 1966. Degradation of the sterol ring structure in the intestine, and the significance of this for sterol balance studies. *Gastroenterology.* **51:** 596–597.

15. Grundy, S. M., E. H. Ahrens, and G. Salen. 1968. Dietary β-sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies. *J. Lipid Res.* **9:** 374–387.

16. Gustafsson, B. E. 1948. Germfree rearing of rats: General technique. *Acta Pathol. Microbiol. Scand.* Suppl. 78, 1–130.

17. Gustafsson, B. E. 1959. Lightweight stainless steel systems for rearing germfree animals. *Ann. N. Y. Acad. Sci.* **78:** 17–28.

18. Lowry, O. H., N. J. Rosebrough., A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

19. Gustafsson, B. E., and A. Norman. 1969. Influence of the diet on the turnover of bile acids in germ-free and conventional rats. *Br. J. Nutr.* **23:** 429–442.

20. Shefer, S., S. Hauser, V. Lapar, and E. H. Mosbach. 1972. HMG CoA reductase of intestinal mucosa and liver of the rat. *J. Lipid Res.* **13:** 402–412.

21. Borgström, B. 1969. Quantification of cholesterol absorption in man by fecal analysis after the feeding of a single isotope-labeled meal. *J. Lipid Res.* **10:** 331–337.

22. Quintão, E., S. M. Grundy, and E. H. Ahrens, Jr. 1971. Effects of dietary cholesterol on the regulation of total body cholesterol in man. *J. Lipid Res.* **12:** 233–247.

23. Madsen, D., M. Beaver, L. Chang, E. Bruckner-Kardoss, and B. Wostmann. 1976. Analysis of bile acids in conventional and germfree rats. *J. Lipid Res.* **17:** 107–111.

24. Danielsson, H., and J. Sjövall. 1975. Bile acid metabolism. *Ann. Rev. Biochem.* **44:** 233–253.

25. Balasubramaniam, S., K. A. Mitropoulos, and N. B. Myant. 1973. Evidence for the compartmentation of cholesterol in rat liver microsomes. *Eur. J. Biochem.* **34:** 77–83.

26. Mitropoulos, K. A., N. B. Myant, G. F. Gibbons, S. Balasubramaniam, and B. E. A. Reeves. 1974. Cholesterol precursor pools for the synthesis of cholic and chenodeoxycholic acids in rats. *J. Biol. Chem.* **249:** 6052–6056.

27. Cronholm, T., A. L. Burlingame, and J. Sjövall. 1974. Utilization of the carbon and hydrogen atoms of ethanol in the biosynthesis of steroids and bile acids. *Eur. J. Biochem.* **49:** 497–510.